

REMARKS

Summary of Claim Amendments

Claims 4 and 13 are amended to incorporate the subject matter of claims 5, 9 and 10, and 14, 18 and 19, respectively. Claims 4 and 13 are further amended to recite the rate of flow of ethanol, and the type of nozzle used, with support in the present specification at, for example, page 8, lines 17-19, and page 9, lines 27-28, respectively. Applicants also submit herewith a Non-Patent Literature document, Ohmoto et al. (J. Pharmaceutical Science, Vol. 92, No. 2, pp. 371-380, February 2003), to explain the use of a v-shaped nozzle.

Claims 5, 9, 10, 14, 18 and 19 are canceled without prejudice or disclaimer.

No new matter is added. Accordingly, Applicants respectfully request entry and consideration of the Amendment. Upon entry of the Amendment, claims 4, 8, 13, 17 and 20-22 will be pending in the application. Claims 13 and 17 are withdrawn from consideration.

Request for Confirmation of Filing Date of Accepted Drawings

It appears that the Examiner inadvertently failed to indicate the filing date of the Drawings, filed December 9, 2005, which are indicated as accepted in the Office Action Summary dated January 7, 2009. Accordingly, Applicants respectfully request the Examiner to confirm the filing date of the accepted Drawings.

Response to Claim Rejections Under 35 U.S.C. § 102(b)

Claims 4, 5 and 8-10 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Hanna (U.S. Patent No. 6,063,138); and, claims 4, 5 and 9 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Reverchon (Powder Technol., 114, pp. 17-22).

Without acquiescing to the merits, claims 5, 9 and 10 are canceled.

Applicants respectfully traverse the above identified § 102(b) rejections, at least for the reasons below.

In the Office Action dated September 8, 2009, the Examiner considers that the experimental evidence included in the Rule 132 Declaration is not a true side-by-side comparison of Applicants' and Hanna's invention because not enough details of the apparatus used and the process parameters have been shown.

In response, claim 4 is amended to specify parameters, such as ethanol flow rate, used in Applicants' invention. Claim 4 is further amended to incorporate the subject matter of claims 9 and 10, thereby further defining the supercritical fluid and modifier.

Additionally, Applicants submit that the use of a v-shaped nozzle in Applicants' invention does not effect the final product. Moreover, the v-shaped nozzle used in Applicants' invention is different from the co-axial nozzle described at column 8, lines 23-37, and column 9, lines 6-8, of Hanna.

Accordingly, Applicants submit that neither Hanna nor Reverchon teaches each and every element of claim 4, and the presently claimed product, formed using the process of claim 4, is distinguishable over the product of Hanna and Reverchon, based on the results shown in the Rule 132 Declaration filed May 7, 2009. Therefore, claim 4 is patentable over Hanna and

Reverchon. Claim 8 is also patentable, at least by virtue of its dependence from claim 4. In view of the above, Applicants respectfully request reconsideration and withdrawal of the § 102(b) rejections of the present claims.

Response to Claim Rejection Under 35 U.S.C. § 103(a)

Claims 4, 5, 8-10 and 20-22 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Hanna and Reverchon in view of Yianneskis (U.S. Patent No. 5,975,076) and Szabo (Journal of Crystal Growth, Vols. 237- 239, Part 3, pp. 2240-2245).

Without acquiescing to the merits, claims 5, 9 and 10 are canceled.

Applicants traverse the § 103(a) rejection, at least because neither Yianneskis nor Szabo cures the above discussed deficiency in Hanna and Reverchon.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the §103(a) rejection of claims 4, 8 and 20-22.

Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

AMENDMENT UNDER 37 C.F.R. § 1.111
U.S. Appln. No.: 10/560,169

Attorney Docket No.: Q91343

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

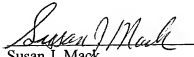
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Pulmonary Gene Delivery by Chitosan–pDNA Complex Powder Prepared by a Supercritical Carbon Dioxide Process

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ABSTRACT: Chitosan–plasmid DNA (pDNA) complex powders as a pulmonary gene delivery system were prepared with a supercritical carbon dioxide (CO₂) process and their *in vivo* activity was evaluated. The powders with mannitol as a carrier were prepared by dispersing aqueous solutions of a luciferase expression plasmid driven by the cytomegalovirus promoter (pCMV–Luc) with or without chitosan as a cationic vector in a supercritical CO₂/ethanol admixture. The supercritical CO₂ process with a V-shaped nozzle successfully produced chitosan–pDNA powders. The addition of chitosan suppressed the degradation of pCMV–Luc during the supercritical CO₂ process and increased the yield of powders. The luciferase activity in mouse lung was evaluated after pulmonary administration of the powders or pCMV–Luc solutions. The chitosan–pDNA powders increased the luciferase activity in mouse lung compared with pCMV–Luc powders without chitosan or pCMV–Luc solutions with or without chitosan. The chitosan–pDNA powder with an N/P ratio = 5 increased the luciferase activity to 2700% of that of the pCMV–Luc solution. These results suggest that gene powder with chitosan is a useful pulmonary gene delivery system. © 2003 Wiley-Liss, Inc. and the American Pharmaceutical Association *J Pharm Sci* 92:371–380, 2003

Keywords: plasmid DNA; chitosan; supercritical carbon dioxide; dry powder; gene delivery system

INTRODUCTION

The recent advances in gene technology have opened the gate for us to step forward into the new field of medical technology, gene therapy. The respiratory system, from the nasal cavity to alveoli, is the site suitable for this gene therapy because direct access of a gene delivery system via the airway is possible. There have been many studies to deliver genes to the lungs for treatment of pulmonary diseases such as lung cancer, cystic fibrosis (CF), and allergen-induced airway hyperresponsiveness. For example, intratracheal delivery of the genes for interleukin-12 or interferon γ has been shown to reduce the severity of allergen-

induced airway hyperresponsiveness in rodent models.¹ CF is the most widely studied target for lung-directed gene therapy. In this decade, proof of principle *in vitro* and then in animal models *in vivo* has been followed by numerous clinical studies using both viral and nonviral vectors to transfer normal copies of the gene to the lungs and noses of CF patients. Gene therapy for CF remains the most promising possibility for curative rather than symptomatic therapy.²

The success in gene therapeutic strategies depends on an efficient system for the delivery of nucleic acid into the target cells. Both viral and nonviral gene delivery systems have been used in clinical trials to treat maladies such as CF and several forms of cancer. The majority of gene delivery methods have involved primarily adenoviral or liposomal vectors.³ Cationic polymers also have the potential for DNA complexation, and it is recognized that they may be useful as nonviral

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vectors for gene delivery. Many cationic polymers, such as chitosan,^{4,5} polylysine,⁶ and poly(ethyleneimine),⁷ as well as cationic liposomes⁸ and cationic microsphere⁹ have been examined and reported as DNA delivery systems. Richardson et al.⁴ examined the potential of low molecular mass chitosan as a DNA delivery system. They observed that the highly purified chitosan fractions used were neither toxic nor hemolytic and that they have the ability to complex DNA and protect against nuclease degradation. Furthermore, low molecular weight chitosan can be administered intravenously without liver accumulation.

Although aqueous solution instillation appears to be the most common way for gene delivery to the airway, there have been several other formulations reported. Use of organic solvents such as 15% di-*n*-propylsulfoxide or perfluorochemical liquids have been reported to increase gene expression in the lungs.⁹ Brown et al. prepared propellant-driven aerosols by suspending lyophilized lipofectin-DNA plasmid complex in dimethylether propellant.¹⁰ Nebulization is one of the practical systems for the administration of nonviral gene delivery systems. Stribling et al. demonstrated that aerosol administration of a chloramphenicol acetyltransferase (CAT) expression plasmid complexed to cationic liposomes produces high-level, lung-specific CAT gene expression in mice *in vivo* with no apparent treatment-related damage.¹¹ However, it is the case as reported by Eastman et al. that the free plasmid DNA (pDNA) was degraded rapidly during aerosolization, although complexed pDNA was largely protected.¹² Birchall et al. also reported that the process of jet nebulization adversely affected the physical stability of lipid-pDNA complexes. The physical stability and biological activity of nebulized lipid-pDNA complexes can be improved by inclusion of a condensing polycationic peptide such as protamine.¹³

Another promising system for pulmonary gene delivery is dry powder. However, the information on the usefulness of dry powder as a pulmonary gene delivery system is very limited. Freeman and Niven administered 200 µg of a luciferase expression plasmid driven by the cytomegalovirus promoter (pCMV-Luc) as a spray-dried pCMV-Luc/trehalose (1:9) powder into rat lung through the trachea to examine the effect of insufflated pDNA on the transfection of lung tissue. Unfortunately, no response to the insufflated pDNA powder could be obtained.¹⁴

Supercritical fluid technology offers the possibility to produce dry powder formulations suitable for inhalation or needle-free injection.¹⁵ Supercritical carbon dioxide (CO₂) has been employed for the preparation of lactose,¹⁶ steroids,¹⁷ protein powder,¹⁸ biodegradable microsphere,^{19,20} and liposomes.²¹ Tserstivas et al. described the first application of a process involving supercritical CO₂ for the production of pDNA-loaded particles. They used the technique of solution-enhanced dispersion by supercritical fluids (SEDS) to coformulate the 6.9 kb plasmid pSVβ with mannitol as the excipient.¹⁶ However, the *in vivo* efficacy of the pDNA powders has not been reported.

The cationic vectors are expected to increase the integrity of pDNA during processing in a supercritical fluid as well as increase the transfection efficacy. In the present study, we prepared gene powders using pCMV-Luc as a reporter gene and a low molecular weight chitosan ($M_w = 3000-30,000$) as a cationic vector with supercritical CO₂. The obtained chitosan-pDNA powders were administered to mice lungs. The transfection efficiency of the powders was compared with that of pDNA solutions and pDNA powders without the cationic vector.

MATERIALS AND METHODS

Materials

A pDNA pCMV-Luc, constructed by subcloning the Hind III/Xba I firefly luciferase cDNA fragment from the pGL3-control vector into the polylinker of the pCDNA3 vector was donated by Prof. M. Hashida, Kyoto University.²² The pDNA was amplified in the DH5 strain of *Escherichia coli* and purified using a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany). The concentration of pDNA was measured by ultraviolet (UV) absorption at 260 nm. The pDNA purity was assessed by measuring the A_{260}/A_{280} ratios.

Water-soluble chitosan (nominal $M_w = 3000-30,000$; Wako Pure Chemical Industries Ltd., Osaka, Japan), mannitol (Wako Pure Chemical Industries Ltd.), and sodium acetate (Kishida Chemicals Ltd., Osaka, Japan) were used as a nonviral vector, a dry powder carrier, and an additive, respectively.

A luciferase assay system PicaGene[®] was purchased from Toyo Ink, Tokyo, Japan. The other reagents and solvents used were of analytical grade.

Preparation of Chitosan-pDNA Complex Powder with Supercritical Carbon Dioxide

The apparatus for preparation of the chitosan-pDNA complex powder (Figure 1) was composed of two CO₂ pumps (SCF-Get, the maximum flow rate was 10 mL/min each), a modifier pump (PU-1580), an aqueous solution pump (PU-1580), an oven (GC353B, GL Sciences Inc., Tokyo, Japan), and a back pressure regulator (SCF-Bpg; the pressure regulation range was 1–50 MPa) which were manufactured and assembled by JASCO Company, Tokyo, Japan, except for the oven. The dry powder preparation vessel had a unique V-shaped nozzle designed by us (Figure 1). The CO₂ flowed at a rate of 5.7 g/min and was admixed with ethanol flowing at a rate of 0.665 mL/min, which enabled water to be miscible with the nonpolar CO₂ in the mixing column. The admixture was flowed into the particle formation vessel (2.0 cm id and 14 cm height; 35°C and 15 MPa) through one end of the V-shaped nozzle. Water flowed into the particle formation vessel at a rate of 0.035 mL/min through the other end of the V-shaped nozzle. An aqueous chitosan-pDNA complex solution (0.4 mL) was injected into the water stream through a manual injector. Half an hour after the injection, the flow of water and ethanol was stopped and CO₂ was flowed for additional 90 min to completely remove the water and ethanol in the vessel. The dry powder was har-

vested from the depressurized vessel. The formulation compositions are listed in Table 1. The yield was calculated for amount of product but not for amount of DNA.

The aqueous chitosan-pDNA complex solutions were prepared by admixing pCMV-Luc (120 µg) and chitosan (142, 283, and 566 µg) in 0.4 mL of purified water to make N/P ratios 2.5, 5, and 10. The N/P ratios were calculated on the basis of chitosan nitrogen per pCMV-Luc phosphate. Mannitol was added to the chitosan-pDNA complex solutions after the complex solutions were left for 30 min at a room temperature.

The pDNA powder with sodium acetate was prepared by referring to the report by Tservistas et al.¹⁵

Evaluation of Particle Size Distribution and Particle Shape

The particle size distribution of the obtained powder was measured with a laser micron sizer LMS-30 (Seishin Enterprise Company Ltd., Tokyo, Japan) based on laser diffraction. We dispersed the powder into a laser beam directly from the apparatus used for intratracheal administration in mice (Figure 2). An electron microscope (JSM-T20, JOEL, Tokyo, Japan) was used to observe the particle shape.

Determination of pCMV-Luc Integrity by Electrophoresis

The powder was dissolved in purified water, and the resulting solution containing 0.06 µg of pCMV-Luc was loaded on a 0.6% agarose gel containing ethidium bromide. Electrophoresis was carried out with a current of 100 V for 2 h in TAE running buffer.

In Vivo Pulmonary Transfection Study in Mice

Female ICR mice weighing ~20 g were anesthetized with pentobarbital (50 mg/kg, ip) and secured on their backs on a board during the experiments. The trachea was exposed, and a 3.0-cm length of PE-60 polyethylene tubing was inserted to a depth of 1.0 cm through an incision made between the fifth or sixth tracheal rings caudal to thyroid cartilage.

On intratracheal administration of the solution, 50 µL of the solution containing 3 µg of pCMV-Luc with 0, 1.8, 3.6, 7.1, or 14 µg of chitosan

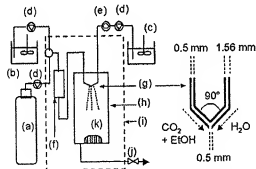


Figure 1. Schematic diagram of the apparatus for preparation of the chitosan-pDNA complex powder with supercritical carbon dioxide: (a) CO₂ cylinder, (b) ethanol, (c) water, (d) pump, (e) manual injector for aqueous pCMV-Luc solution with or without chitosan, (f) mixing column, (g) V-shaped nozzle, (h) particle formation vessel, (i) oven, (j) back pressure regulator, and (k) product. The V-shaped nozzle has an outer diameter of 1.56 mm and an inner diameter of 0.5 mm.

Table 1. pDNA Powders Prepared With Supercritical Carbon Dioxide

Formulation Title	Formulation Composition (mg) ^a				Yield (%) ^b	Mean Particle Diameter (μm)
	Mannitol	pCMV-Luc	Chitosan	Sodium Acetate		
pDNA _{dp} (N/P = 0)	60.0	0.120	0	0	68.5	12.7
Chitosan-pDNA _{dp} (N/P = 2.5)	59.7	0.120	0.142	0	81.1	13.2
Chitosan-pDNA _{dp} (N/P = 5)	59.6	0.120	0.283	0	82.2	12.2
Chitosan-pDNA _{dp} (N/P = 10)	59.3	0.120	0.566	0	79.9	12.2
AcNa-pDNA _{dp}	57.7	0.120	0	2.16	62.5	13.3

^aNominal amounts of ingredients dissolved in 400 μL of water injected in the supercritical carbon dioxide.

^bYield = (amount of powder recovered from the particle formation vessel)/(amount of mannitol, pCMV-Luc, chitosan, and/or sodium acetate injected in the vessel) × 100.

in a microsyringe was administered into the mouse trachea.

We administered the pCMV-Luc powders through the mouse trachea using the apparatus for mice (Figure 2), which was assembled by modifying the apparatus for pulmonary administration in rats.²⁸ The powder (1.5 mg) was put in a disposable tip and dispersed in the mouse trachea by releasing air (0.25 mL) compressed in a syringe by opening a three-way stopcock connecting the disposable tip and the syringe.

The mice were sacrificed 3, 6, or 9 h after administration by exsanguination from the aorta, and the whole lung was carefully removed. The lung was washed twice with ice-cold phosphate buffered saline (PBS) and homogenized with a lysis buffer. The lysis buffer consisted of 0.1 M Tris/HCl buffer (pH 7.8) containing 0.05% Triton X-100 and 2 mM EDTA. The volume of the lysis buffer added was 4 mL/g tissue. After three cycles of freezing and thawing, the homogenate was centrifuged at 15,000 rpm for 5 min at 4°C. Twenty microliters of each supernatant were subjected to the luciferase assay with PicaGene[®], and the light produced was immediately measured with a luminometer (Lumat LB 9507, EG & G Berthold, Bad Wildbad, Germany) for 10 s.

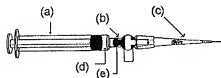


Figure 2. Apparatus for pulmonary administration of dry powder: (a) 1-mL syringe, (b) 3-way stopcock, (c) disposable tip with dry powder, (d) compressed air, and (e) administration handle.

Statistical Analysis

Statistical differences in luciferase activity were examined using a one-way analysis of variance (ANOVA) followed by least significant difference test. The significance level was set at $p < 0.05$.

RESULTS

Characterization of Chitosan-pDNA Complex Powder Prepared with Supercritical Carbon Dioxide

The supercritical CO₂ process with a V-shaped nozzle successfully prepared chitosan-pDNA complex powders. The scanning electron microscope observation showed that the powders were of rectangular shape (Figure 3), with the short axis <10 μm and long axis >10 μm. The mean particle diameters of the powders measured by the laser micron sizer were 12–13 μm (Table 1). The yields of pCMV-Luc/mannitol and pCMV-Luc/sodium acetate/mannitol powders were <70% (DNA_{dp} and AcNa-DNA_{dp} in Table 1,

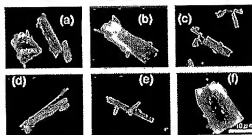


Figure 3. Scanning electron micrographs of pDNA powders with mannitol as a carrier: (a) mannitol alone, (b) pDNA_{dp}, (c) chitosan-pDNA_{dp} (N/P = 2.5), (d) chitosan-pDNA_{dp} (N/P = 5), (e) chitosan-pDNA_{dp} (N/P = 10), and (f) AcNa-pDNA_{dp}.

respectively). In contrast, the powders with chitosan (chitosan-pDNA_{sp}) were harvested at yields of ~80% (Table 1).

During the supercritical CO₂ process, the degradation of pCMV-Luc was observed for the powder without chitosan (lane 2 in Figure 4). The addition of chitosan at an N/P ratio = 2.5 reduced the degradation of plasmid on the gel. Increasing the N/P ratio to 5 or 10 resulted in the degradation band disappearing from the gel (lanes 4 and 5 in Figure 4). Sodium acetate also suppressed the degradation of pCMV-Luc during the supercritical CO₂ process (data not shown), as reported by Tservistas et al.¹⁵

Determination of pCMV-Luc Dose for the Transfection Study

At the beginning of the transfection study, we determined the optimum dose of pCMV-Luc to administer in the mouse lung to evaluate the transfection efficacies of the pCMV-Luc delivery systems. Doses of 1, 3, 10, or 30 µg of the pCMV-Luc dissolved in 50 µL of purified water was administered in the mouse lung for 6 h. The luciferase activities for 3, 10, and 30 µg of the pCMV-Luc were not significantly different among them but were significantly larger than that for 1 µg of the pCMV-Luc (Figure 5; the luciferase activities were normalized by the averaged luciferase activity determined for the 3 µg group and expressed in percent). This result suggested that the pCMV-Luc dose of 3 µg was adequate to express maximum transfection of pCMV-Luc in water, and we adopted this dose for the entire study.

We found some day-to-day variation in the standard curves for the luciferase activity assay. Below, we used 4 or 5 mice administered 3 µg of the

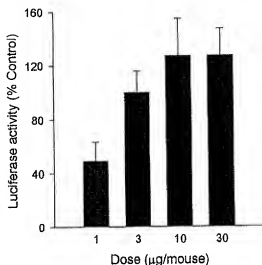


Figure 5. The effect of pCMV-Luc dose on luciferase activity in mouse lungs. Each dose of pCMV-Luc was dissolved in 50 µL of purified water. The luciferase activity assayed 6 h after the pulmonary administration. The error bars indicate standard error (SE) for 3–5 experiments. Statistical significance ($p < 0.05$) was observed for 1 versus 3 µg, 1 versus 10 µg, and 1 versus 30 µg.

pCMV-Luc dissolved in 50 µL of water for 6 h in every set of experiments as a control group. The luciferase activities were normalized by the averaged luciferase activity determined for the control group and expressed in percent.

Luciferase Activity for pCMV-Luc Solution Systems

The potency of chitosan (N/P = 1.25, 2.5, 5, and 10) as a vector was examined with aqueous solution systems. The addition of chitosan at N/P = 1.25 did not affect the luciferase activity. The increase in the N/P ratio resulted in the increased luciferase activity in the mice lungs. The maximum activity observed for the chitosan-pDNA system at an N/P ratio of 2.5 was 360% of that of the pCMV-Luc solution (data not shown).

The luciferase activities for mice administered aqueous pCMV-Luc solution or chitosan-pDNA (N/P = 2.5) solution were maximized at 6 h after the administration and were significantly higher than those at 3 and 9 h after the administration (Figure 6). The luciferase activities for mice administered chitosan-pDNA (N/P = 2.5) solution



Figure 4. The effect of chitosan on the integrity of pCMV-Luc in powders prepared with supercritical carbon dioxide: Lane 1, aqueous pCMV-Luc solution; Lane 2, pDNA_{sp}; Lane 3, chitosan-pDNA_{sp} (N/P = 2.5); Lane 4, chitosan-pDNA_{sp} (N/P = 5); and Lane 5, chitosan-pDNA_{sp} (N/P = 10). Key: (sc) supercoiled; (lin) linear; (oc) open-circular.

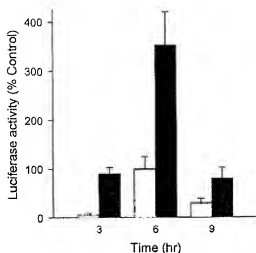


Figure 6. The luciferase activity after the pulmonary administration of pCMV-Luc solutions with time. Key: (□) pulmonary administration of pCMV-Luc (3 μ g) dissolved in 50 μ L of water; (■) pulmonary administration of chitosan-pDNA (N/P = 2.5, 3 μ g pCMV-Luc equivalent) dissolved in 50 μ L of water. The error bars indicate SE for 3–7 experiments. Statistical significance ($p < 0.05$) was observed for intravenous versus chitosan-pDNA and pCMV-Luc versus chitosan-pDNA at 3, 6, and 9 h.

were significantly higher than those administered aqueous pCMV-Luc solution at the time points.

Optimum N/P Ratio for Chitosan-pDNA Powder System

The chitosan-pDNA powders at various N/P ratios were administered to the mouse lung and the luciferase activities at 9 h were assayed. Even the powder without chitosan [pDNA_{dp} (N/P = 0)] increased the luciferase activity to 360% of that of the control (Figure 7), which is comparable to the highest activity attained by the chitosan-pDNA (N/P = 2.5) solution (Figure 6). The powder chitosan-pDNA_{dp} (N/P = 5) showed luciferase activities (1.1×10^6 RLU/mg protein) significantly higher than the other chitosan-pDNA powders.

Luciferase Activity for pCMV-Luc Powder Systems with Time

The efficacies of the powders chitosan-pDNA_{dp} (N/P = 5) and AcNa-pDNA_{dp}, which successfully suppressed pCMV-Luc degradation during the supercritical CO₂ process, were evaluated by

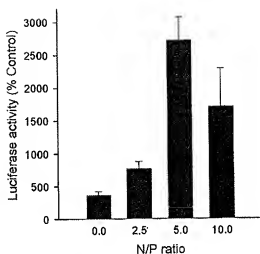


Figure 7. The effect of N/P ratio on the luciferase activity expressed in mouse lungs 9 h after the pulmonary administration of pCMV-Luc powders. The error bars indicate SE for 4–8 experiments. Statistical significance ($p < 0.05$) was observed for N/P = 0 versus 5, N/P = 0 versus 10, and N/P = 2.5 versus 5.

administering them to mouse lung using the apparatus shown in Figure 2. The luciferase activity in the mouse lung was increased with time after the pulmonary administration of chitosan-pDNA_{dp} (N/P = 5) and reached 2700% of that of the control at 9 h (Figure 8). (The control mice were sacrificed 6 h after the administration of pCMV-Luc solution.) The luciferase activity was reduced to 91% of that of the control at 24 h after the administration of chitosan-pDNA_{dp} (N/P = 5). The pCMV-Luc powder stabilized with sodium acetate (AcNa-pDNA_{dp}) also increased the luciferase activity with time; however, the maximum activity observed at 9 h was only 305% of the control. The luciferase activities observed for chitosan-pDNA_{dp} (N/P = 5) was significantly higher than those for AcNa-pDNA_{dp} at all time points.

DISCUSSION

Intravenous (iv) administration of pDNA can deliver pDNA to the lungs. Niven et al. examined the tissue biodistribution and expression of pDNA-DOTIM/cholesterol complexes expressing CAT after iv injection in mice.⁷ The radiolabeled pDNA-DOTIM/cholesterol complexes were

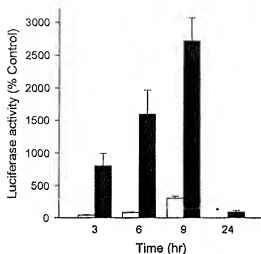


Figure 8. The luciferase activity after the pulmonary administration of pCMV-Luc powders with time. Key: (□) AcNa-pDNA_{dp}; (■) chitosan-pDNA_{dp} (N/P = 5); (*) the 24-h data for AcNa-pDNA_{dp} are not available. The error bars indicate SE for 3–8 experiments. Statistical significance ($p < 0.05$) between chitosan-pDNA_{dp} (N/P = 5) and AcNa-pDNA_{dp} was observed at 3, 6, and 9 h.

initially deposited in the lungs. However, the radioactivity in lung decreased rapidly and that in the liver subsequently increased. The majority of pDNA incubated in blood was degraded within 2 h. In general, pulmonary delivery of pDNA via an iv route has concerns such as (a) the pDNA will distribute to several organs other than the lungs, (b) pDNA will be degraded in plasma before reaching the lungs, and (c) the patient will suffer from pain from the needle stick.

Inhalation of gene delivery systems provides solutions to these concerns. Some basic studies administered pDNA solutions directly to the lung through the trachea. In 1995, Tsan et al. demonstrated that tracheal insufflation of pDNA expressing CAT, driven by the CMV promoter, resulted in efficient and selective transfection of the lungs in rats.²⁴ When the simian virus 40 (SV40) promoter was used, there was no detectable transfection. These results suggested that the SV40 promoter is not suitable for pulmonary delivery of pDNA. In the present study we employed pCMV-Luc as a reporter pDNA to evaluate the pulmonary gene delivery systems.

There are three main delivery systems used for aerosol inhalation in humans; they are, pressurized metered-dose inhalers, nebulizers, and dry

powder inhalers (DPI).²⁵ Among these, DPI appears to be the most promising for future use.^{25,26} In the present study, we produced gene powders using the supercritical CO₂ process because this process is suitable for an early stage of study to produce gene powders with a small amount of pDNA and may be easy to scale up in future.

The findings of the present study revealed that the gene powder with the cationic polymer is an excellent gene delivery system to the lungs. The benefits of the chitosan-pDNA powders examined are summarized as follows: (a) the addition of chitosan suppressed the degradation of pCMV-Luc during the supercritical CO₂ process, (b) the addition of chitosan increased the yield of powders, and (c) the chitosan-pDNA powders increased the luciferase activity in the mouse lung compared with pCMV-Luc powders without chitosan or pCMV-Luc solutions with or without chitosan. We did not determine the composition of the powders obtained in the present study because the amount of the powders was limited. We collected the effluent ethanol/water mixture from the back pressure regulator. No pCMV-Luc was detected for the effluent on the electrophoresis gel, suggesting that a large part of the pCMV-Luc injected in the vessel was recovered in the powders.

Tservistaa et al. reported the powders of pSVβ with mannitol as the excipient prepared by the supercritical CO₂ process with a three-channelled coaxial nozzle.¹⁵ A high degradation of the pDNA during powder formation was observed because of a pH drop by the formation of carboxylic acid in the CO₂/isopropanol/water system. The addition of sodium acetate to the aqueous-feed solution successfully increased the recovered supercoiled proportion from 7 to 80%.¹⁵ They concluded this success resulted from the reduced pH drop by sodium acetate. The addition of sodium acetate suppressed the degradation of pCMV-Luc during the supercritical CO₂ process with the V-shaped nozzle in the present study. This result suggests that the observed degradation of pCMV-Luc was attributed to the pH drop during the supercritical CO₂ process rather than the physical stress by spraying the pCMV-Luc solution through the V-shaped nozzle.

In the present study, the addition of chitosan at an N/P ratio of 5 or 10 suppressed the pCMV-Luc degradation detectable on agarose gel. It is known that the complexation of pDNA with cationic vectors stabilizes the pDNA against the DNase⁴ or physical stress such as sonication.^{12,13} The

complexation with the cationic vector may protect the pDNA against chemical degradation processes. Another possibility of chitosan acting as a pDNA protectant against chemical degradation may relate to its buffering capacity. Chitosan is a weak base with its pK_a value reportedly at 7.7.⁵ Chitosan may reduce the pH drop by the formation of carboxylic acid in the present CO_2 /ethanol/water system as does sodium acetate.

Cationic liposomes have been used as potent vectors for gene delivery. However, it has been reported that tracheal insufflation of pDNA resulted in transfection of rat lungs to the same extent as insufflation of the pDNA-cationic liposome complex.²⁴ When 200 μ g of pCMV-Luc was administered into rat lung as a complex with DC-Chol/DOPE (1:1) liposome, the luciferase activity in the lung was almost the same as that in the lung administered pCMV-Luc alone. The addition of 1% sodium glycocholate to the liposome solution was required to increase the luciferase activity.¹⁴ By referring to these reports, we intratracheally administered an aqueous pDNA solution, rather than a pDNA-liposome solution, to the control group in the present study.

To better understand these observations, the *in vitro* gene transfer of pDNA in the presence and absence of cationic liposome and the effect of surfactant on the gene transfer was investigated.²⁷ The results revealed that although surfactants including dipalmitoyl phosphatidylcholine have no effect on *in vitro* gene transfer by pDNA alone, they markedly inhibited cationic liposome-mediated gene transfer. The same kind of observation that pulmonary surfactant lipids and proteins inhibit transfection with pDNA-cationic liposome complexes and may therefore represent a barrier to gene transfer in the lung was reported by other groups.²⁸ Although we have not compared the pDNA powders reported here with any liposomal-pDNA systems, a cationic polymer such as chitosan seems to be a more suitable vector than a cationic liposome for pulmonary gene delivery.

The gene powders examined in the present study have several advantages over the gene solutions as pulmonary gene delivery systems. The physical integrity of pCMV-Luc in pDNA_{dp} (N/P = 0) was decreased, as shown in Figure 4, which would negatively affect the gene expression. Even the powder without chitosan [pDNA_{dp} (N/P = 0)] showed a higher luciferase activity than the control pDNA solution and was as effective as the most potent chitosan-pDNA solution (N/P = 2.5) examined in the present study. The mechanism

should be identified in future studies; however, one of the possible reasons may be attributed to the high concentration of the gene. The gene concentration in the lungs, where the powder deposited, would be much higher compared with where a gene solution is delivered because the powder would dissolve in a small volume of water on the inner surface of the lungs. A high gene concentration may result in lower degradation of the gene in the lungs or during the transfection process. The addition of chitosan in the powder may result in a more concentrated gene delivery and excellent transfection. An N/P ratio 2.5 was sufficient for the aqueous pCMV-Luc solutions to maximize the luciferase expression in the lungs, whereas the N/P ratios of 5 and 10 were more effective than that of 2.5 for powders. This result may relate to the physical integrity of pCMV-Luc. The N/P ratio of 2.5 was not enough to prevent the degradation of pCMV-Luc; whereas the N/P ratios of 5 or 10 almost completely prevented the degradation. However, the finding that the powder with an N/P ratio of 5 gave luciferase activity higher than that with an N/P ratio of 10 suggests that the physical integrity of pCMV-Luc is not the sole explanation for the increased luciferase activity.

The luciferase activity in the lung was maximized at 6 h after the administration of pDNA solutions, whereas it was at 9 h after the administration of pDNA powders. We previously reported that insulin administered to rat lungs decreased the blood glucose level for a longer period than insulin in solution.²⁹ The possible reason of longer action time may be attributed to the time for powder dissolution and/or avoidance of phagocytic clearance from the lung.³⁰ However, the luciferase activity was reduced to almost the control level in 24 h even with chitosan-pDNA_{dp} (N/P = 5). We are going to identify the reason of reduction in luciferase activity to produce longer acting pDNA powders.

The design of the nozzle directly affects the performance of the supercritical CO_2 process. When the CO_2 /ethanol admixture and aqueous solution were introduced in the particle formation vessel through two parallel straight nozzles, no powder was obtained. The V-shaped nozzle would enhance the dispersion of the water into the CO_2 /ethanol admixture at the point where the two streams mix.

The chitosan-pDNA complexes were prepared in aqueous solutions. Because water is not soluble in pure supercritical CO_2 , ethanol, which is miscible with water as well as supercritical CO_2 , was

added as a modifier to help supercritical CO_2 remove the water. It was reported that the single phase vapor region for the CO_2 /ethanol/water system on the phase diagram is very narrow.³¹ The flow rates of CO_2 , ethanol, and water were arbitrarily set to 5.7 g/min, 0.665 mL/min, and 0.035 mL/min, respectively, in the present study because the amount of water miscible in the CO_2 /ethanol admixture appeared to be very limited. The measured equilibrium compositions for ethanol/water/ CO_2 mixtures at 35°C and 13.6 or 17.0 MPa are available in the literature.³² The molar composition of the fluid phase of the present study operated at 35°C and 15 MPa was CO_2 /ethanol/water = 0.907:0.080:0.013. This composition falls in the homogeneous region at 35°C and 13.6 or 17.0 MPa (Figure 9). The conditions of the supercritical CO_2 process, such as these flow rates, temperature, and pressure, should be optimized in the future because these conditions would affect the properties of the gene powders, such as particle size, yield, and/or stability.

The aerodynamic diameter markedly affects the efficacy of the powder for inhalation.³⁰ The mean particle diameters of the powders in the present study were 12–13 μm based on the laser diffraction. The aerodynamic diameters were not determined because only a small amount of powders

were available at this stage. In our laboratory, we have prepared insulin powders with mannitol as a carrier by the supercritical CO_2 process as described in this report. The powders had the same shape and size as the pDNA powders shown in Figure 3 and the aerodynamic diameters determined by cascade impactor study were $\sim 3 \mu\text{m}$. This result suggests that although we should determine the diameter in the future, the pDNA powders with mannitol as a carrier also have aerodynamic particle sizes of $\sim 3 \mu\text{m}$, which is suitable for inhalation.

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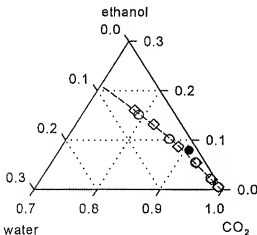


Figure 9. The triangular diagram for ethanol/water/ CO_2 mixtures at 35°C. The measured equilibrium compositions at 17.0 MPa (O) and at 13.6 MPa (◊) were quoted from ref 32. The molar composition of the fluid phase of the present study (15 MPa) is shown with the symbol ●. Ethanol, water, and CO_2 are completely miscible at the region on and above the binodal curve (broken line).